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MOVEMENT OF SODIUM INTO HUMAN PLATELETS

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Summary

Addition of ADP induces platelets in plasma to undergo shape change from a disc to a spiny sphere and to develop adhesiveness, i.e. to aggregate. The aggregation of human platelets by ADP is associated with a net uptake of Na^+. The present experiments demonstrate that the induction of shape change by ADP in acidified or EGTA-treated plasma conditions which inhibit aggregation, is also associated with a movement of Na^+ into platelets. When ADP-induced platelet shape change and aggregation is inhibited by prostaglandin E_1 Na^+ uptake is also blocked. Platelets aggregated by epinephrine do not take up Na^+. In a manner analogous to the effect of ADP, polylysine also induces Na^+ uptake during aggregation. Vasopressin, in a manner analogous to epinephrine, induces aggregation without Na^+ uptake. The increase in platelet Na^+ resulting from ouabain inhibition of Na^+ efflux induces an increase in the aggregation response to ADP and to epinephrine.

Introduction

Effector molecules, e.g., ADP, induce platelet shape change and aggregation when they react with sites on the surface membrane. However, the transduction mechanism between the membrane and activation processes within the platelet is unknown. Most cells maintain ion transmembrane gradients and excitation is associated with the movement of ions down their electrical and chemical gradients. Platelets maintain low intracellular [Na^+] and high [K^+] by means of a ouabain sensitive Mg^{2+}-dependent, $Na^+ + K^+$ stimulated ATPase [1]. Platelets also maintain low levels of Ca^{2+} as evidenced by the presence of microtubules [2]. We and others have shown that ADP-induced aggregation does not involve the movement of Ca^{2+} into the cell [3,4] and, indeed, ADP-induced shape change can take place in the presence of external calcium chelators [5].

On the other hand, aggregation of platelets by ADP is associated with Na^+ influx even after partial dissipation of the Na^+ transmembrane gradient by ouabain [6]. Since epinephrine, which also aggregates platelets, has been shown to induce an uptake of Ca^{2+} [3] an ion-specific, effector-specific, ion flux may be involved in platelet activation.

In the present report we show that Na^+ influx also accompanies ADP-induced platelet shape change and that platelets aggregated by epinephrine do not take up Na^+. Ouabain treatment, which raises platelet (Na^+), leads to increased sensitivity of platelets to both ADP and to epinephrine; an indication that changes in intraplatelet (Na^+) levels may be involved in processes leading to platelet functional change.

Materials and Methods

Human platelet-rich plasma was prepared as previously described [7] by centrifuging citrated blood from healthy donors at 164 × *g* for 15 min. The plasma was incubated with $^{22}NaCl$ and ^{125}I-labeled human serum albumin. Platelets from 1 ml of platelet-rich plasma were sedimented through silicone oil [8], and platelet pellets as well as samples of platelet-free plasma were prepared for radioactivity determinations as previously described [6]. The volume of plasma trapped within the sedimented platelets was estimated from the ^{125}I present in the pellets.

The platelet ^{22}Na radioactivity was determined as the difference between total pellet ^{22}Na radioactivity and ^{22}Na in the trapped plasma: the Na^+ taken up by platelets was calculated on the basis of the specific activity of ^{22}Na in plasma.

Platelet aggregation and shape change were measured photometrically [9] and platelets were counted by phase contrast microscopy; the counts ranged from $2 \cdot 10^8$ to $4 \cdot 10^8$ platelets/ml. In each experiment quadruplicate samples of platelet-rich plasma were taken for determination of ^{22}Na associated with platelets immediately prior to agonist addition and again at 60, 120 and 180 s after adding the agonist. Analysis of variance was used to determine whether the differences among the means were significant. The criteria of Snedecor [10] were used to determine whether the mean values at each sampling time were significantly different ($P < 0.05$) from the mean obtained prior to agonist addition.

Results

The effect of ADP-induced shape change on Na^+ uptake

ADP-induced aggregation was inhibited by lowering the pH of the plasma to 6.5 (5 experiments) or by adding EGTA (10^{-3} M) (6 experiments) to chelate extra-platelet Ca^{2+} [11,12]. Under these conditions the addition of ADP induced a characteristic change in platelet change. During the first 60 s following ADP addition (10^{-5} M), the platelets in acidified plasma took up 5.2 ± 1.4 nequiv./10^8 platelets (mean ± S.E., $n = 20$). Similarly in the presence of EGTA (Fig. 1), the platelets took up 5.8 ± 1.4 nequiv./10^8 platelets (mean ± S.E., $n = 24$). This influx of Na^+ was sustained at 120 and 180 s after ADP induction of shape change.

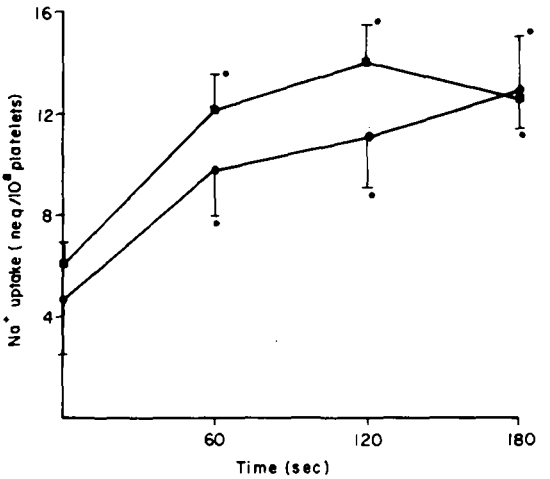

Fig. 1. Effect of ADP-induced shape change on Na^+ uptake. Shape change was initiated by addition of ADP (10^{-5} M) to acidified plasma (titrated with HCl to pH 6.5) (●) and to EGTA-treated plasma (10^{-3} M) (■). The uptake of Na^+ in nequiv./10^8 platelets (shown on the ordinate) was calculated from the ^{22}Na taken up by the platelets and the specific activity of ^{22}Na, in plasma. Time after ADP addition is shown on the abscissae. Vertical bars indicate standard error of the mean (acidified plasma, 5 experiments and EGTA treated plasma, 6 experiments). Analysis of variance (ANOVA) indicated that significant differences ($P < 0.05$) occurred among the Na^+ uptake means of the acid-treated ($F = 3.17$) and the EGTA-treated ($F = 8.17$) plasma. The asterisk indicates a significant difference ($P < 0.05$) of the mean at 60, 120 or 180 s from the mean before addition of agonist.

The effect of epinephrine on Na^+ uptake

The ability of epinephrine to induce Na^+ uptake was compared to that of ADP in 10 separate experiments. In order to insure that the epinephrine

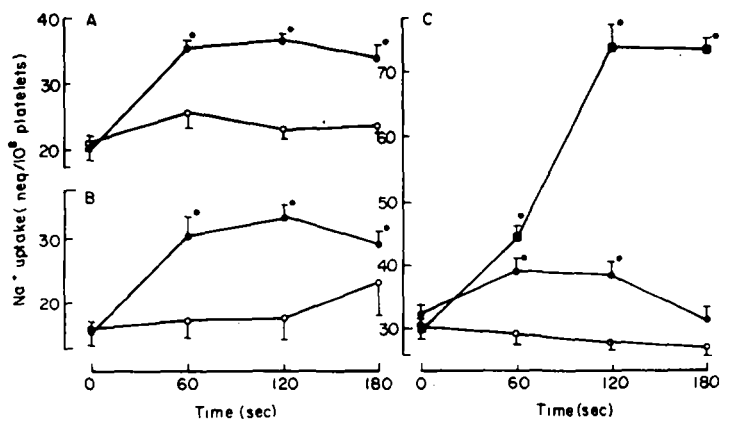

Fig. 2. Na^+ uptake by platelets. Uptake of Na^+ (nequiv./10^8 platelets) is plotted on the ordinate and time after agonist on the abscissa. A. Aggregation initiated by ADP (10^{-5} M) (●——●) or by epinephrine (10^{-5} M) (○——○); mean of 10 experiments. B. Platelet-rich plasma was treated with prostaglandin E_1 (10^{-8} M) (○——○) or with saline (●——●) and ADP (10^{-5} M) was added at zero time; mean of 5 experiments. C. Polylysine (0.1 mg/ml) (■——■), or vasopressin (370 mU/ml) (○——○) or ADP (10^{-6} M) (●——●) was added at zero time to platelet-rich plasma; mean of 4 experiments. Vertical bars indicate the standard error of the mean. The asterisk indicates a significant difference ($P < 0.05$) of the mean after addition of agonist.

response was independent of ADP secretion, granular release was inhibited by incubating the plasma with aspirin (1 mg/ml) (5 experiments). In the remaining 5 experiments epinephrine induced only a primary wave of aggregation indicating the absence of ADP secretion. It was found that the addition of ADP induced a significant uptake of Na^+ in conjunction with aggregation ($F = 8.77$) while no Na^+ uptake was seen with epinephrine-induced primary aggregation ($F = 1.08$) (Fig. 2A).

The effect of ouabain on ADP and epinephrine-induced aggregation

The addition of ouabain (10^{-6} M) to platelet-rich plasma led to a significant rise in platelet Na^+ and to a decrease in K^+ (Fig. 3). However even after 90 min of incubation, platelet Na^+ was still less than plasma Na^+ (i.e., a transmembrane Na^+ gradient persisted). Even though ouabain itself did not induce platelet aggregation, ouabain treatment sensitized the platelets to ADP and to epinephrine. Thus, one hour after incubation of plasma with ouabain (10^{-6} M) at 37°C the extent of aggregation in response to ADP (10^{-6} M) and epinephrine (10^{-6} M) increased, 63 ± 17 and 45 ± 10 percent, respectively. Pretreatment of Platelet-rich plasma with aspirin (1 mg/ml) did not prevent the augmentation of aggregation by ouabain. The effect of ouabain to augment ADP-induced aggregation was dependent on the ouabain concentration and the duration of exposure to ouabain (Fig. 4).

The effect of prostaglandin E_1 on ADP-induced Na^+ uptake

Prostaglandin E_1 (10^{-8} M) added to platelet-rich plasma inhibited shape change and aggregation induced by ADP (10^{-5} M). The inhibition of platelet functional change was also associated with inhibition of Na^+ uptake at 60, 120, and 180 seconds following ADP addition; ($F = 0.70$ for the Prostaglandin E_1-treated and $F = 4.93$ without Prostaglandin E_1) (Fig. 2B).

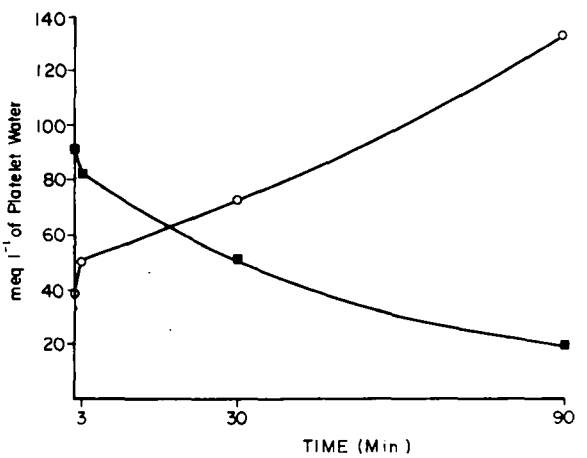

Fig. 3. The effect of ouabain on platelet Na^+ and K^+. Ouabain (10^{-6} M) was incubated with platelet-rich plasma at 37°C. The platelet [Na^+] (o———o) and [K^+] (■———■) were measured by atomic absorption spectrometry and are shown in mequiv./l of platelet water on the ordinate.

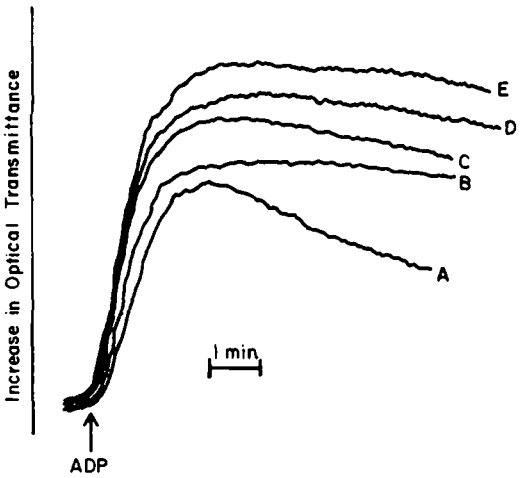

Fig. 4. Effect of ouabain on ADP-induced platelet aggregation. Platelet-rich plasma was incubated with ouabain at 37°C for varying lengths of time and samples were taken for measurement of ADP (10^{-6} M)-induced aggregation. A, Aggregation in the absence of ouabain; B, 30 min after 10^{-6} M ouabain; C, 20 min after 10^{-5} M ouabain; D, 60 min after 10^{-6} M ouabain, and E, 50 min after 10^{-5} M ouabain.

The effect of polylysine and of vasopressin on Na^+ uptake

It has been previously shown that polylysine, a cationic polymer, induces platelet shape change and aggregation [13]. Fig. 2C illustrates that polylysine (0.1 mg/ml)-induced aggregation was associated with a significant Na^+ uptake at 60, 120 and 180 s ($F = 60.2$). On the other hand, vasopressin (340 mU/ml), which also induces platelet aggregation, did not induce Na^+ uptake throughout the course of the aggregation response ($F = 0.07$) (Fig. 2C).

Discussion

Transmembrane movement of ions down their electro-chemical gradient is the basis of excitability for many cell types. Platelets maintain transmembrane gradients for the cations Na^+, K^+ and Ca^{2+}. Dye fluorescence studies have shown that platelets maintain a membrane potential [14] and, using [^{14}C]-thiocyanate distribution we measured this potential as approximately −55 mV [15]. Since we previously demonstrated that ^{45}Ca does not enter platelets during ADP-induced shape change and aggregation [3,4], the inward movement of Ca^{2+} down an electrochemical gradient does not appear to be involved in the activation of platelets by ADP. However, using ^{22}NaCl, we also showed that ADP-induced aggregation is associated with a movement of ^{22}Na into platelets [6]. Furthermore, during the ADP-stimulated response the ^{36}Cl content of platelets did not change [6], and platelet water volume, as measured by ^3H_2O, did not increase [8]. Thus, the observed ^{22}Na influx is compatible with a net inward cation movement.

In the present report we demonstrate that ^{22}Na movement into platelets is also associated with ADP-induced shape change. By comparison, the amount of Na^+ taken up during the first 60 s of ADP (10^{-5} M) stimulated aggregation is

approx. 2—3 times that observed during shape change. The demonstration that Na^+ influx occurs during ADP-induced shape change is a vital link in establishing whether Na^+ influx is a part of the platelet activation mechanism.

As mentioned above, ADP-induced shape change and aggregation does not involve the uptake of Ca^{2+}. On the other hand, epinephrine activation of platelets is clearly associated with Ca^{2+} uptake [4]; and the present results demonstrate that this uptake occurs in the absence of simultaneous Na^+ influx. It has also been shown that verapamil, which inhibits Ca^{2+} transmembrane flux, blocks both epinephrine-induced aggregation and Ca^{2+} uptake, but has no effect on ADP-induced activation [4]. Thus there appears to be a specificity of ion influx (Na^+ or Ca^{2+}) depending on the effector molecule (ADP or epinephrine). This suggestion is consistent with the previous demonstration that these agonists have different activation mechanisms [17].

Support for the concept that Na^+ influx is an integral part of the ADP activation process is provided by the finding that amiloride (known to block Na^+ transfer across toad bladder and epithelial membrane [18]) inhibits ADP-induced aggregation and platelet $^{22}Na^+$ uptake [19]. Furthermore, it was recently reported that amiloride also inhibits thrombin-induced aggregation and blocks the 'depolarization' of the platelet membrane potential, as measured by dye fluorescence [20]. Consequently a sudden increase in intraplatelet Na^+ may be required as an initiating event in platelet activation by ADP and thrombin.

In this connection we previously demonstrated that ouabain treatment leads to an accumulation of platelet Na^+ and loss of K^+ consistent with inhibition of a Na^+ efflux pump. If a net increase in intraplatelet Na^+ however is the sole basis of platelet activation, then ouabain would be expected to stimulate aggregation; we found, as have others, that it does not [22]. Similarly nigericin, and monensin, which also increase platelet Na^+, do not induce aggregation [23]. On the other hand ouabain treatment does lead to increased sensitivity to ADP and to epinephrine; an indication that ouabain may induce a partial activation. This possibility is supported by the interference-microscopy observations of Barnhart et al. [24], showing that ouabain induces platelet shape change. In this connection we have also found that a high concentration (10^{-4} M) of ouabain will induce changes in light scattering by platelets that are consistent with the development of platelet pseudopods [19]. Since shape change has been shown to be associated with an increase in intraplatelet Ca^{2+}, it is possible that ouabain, by effecting an increase in platelet Na^+, could elevate intracellular Ca^{2+} levels. Thus, in a manner analogous to heart muscle, raised Na^+ levels after ouabain treatment could lead to an enhanced Na_i^+—Ca_o^{2+} exchange [25] or, as shown by Steiner, to decreased binding of intraplatelet Ca^{2+} [26].

Although ouabain appears to induce hypersensitivity to ADP and epinephrine, it also decreases the Na^+ transmembrane gradient. Therefore, based on a chemical gradient, decreased ^{22}Na influx would be expected. We found, however, that despite raised platelet Na^+ levels after ouabain, ADP nevertheless induced a further ^{22}Na uptake [6]. Thus it is possible that the driving force for ADP-induced Na^+ uptake is electrogenic in nature, i.e., an inward Na^+ current. On the other hand, the finding of Horne and Simon [14] and of MacIntyre [27] that ADP activation of platelets does not decrease the membrane poten-

tial would be inconsistent with Na^+ influx acting as an inward positive current.

In addition we found that polylysine, which induces platelet shape change and aggregation, also induces ^{22}Na influx. Polylysine, a cationic polymer is presumed to neutralize the negative surface charge of platelets. It has also been shown to inhibit ($Na^+ + K^+$)-stimulated ATPase [28] and to increase the permeability of negatively charged membrane vesicles to Na^+ [29]. Either of these effects could explain increased Na^+ flux. On the other hand, vasopressin-induced aggregation was not found to be associated with Na^+ uptake. In this regard Haslam and Rosson [30] have demonstrated that vasopressin-stimulated platelet function is highly sensitive to extraplatelet Ca^{2+}, as aggregation is much decreased in citrated relative to heparinized plasma. Consequently it is possible that vasopressin, like epinephrine, induces platelet aggregation through uptake of Ca^{2+} rather than through the uptake of Na^+.

Finally, it is well known that prostaglandin E_1-treated platelets do not change shape, aggregate or secrete in response to ADP [21]. In the present experiments we found that prostaglandin E_1 also inhibits ADP-induced Na^+ influx. This finding suggests the possibility that the movement of Na^+ into the platelet occurs subsequent to an ADP-induced destabilizing action, e.g., dephosphorylation of the platelet membrane.

The above results, therefore, lead us to postulate that an increase in Na^+ permeability, induced by ADP and perhaps other effector molecules, e.g., polylysine, is an essential part of the activation mechanism leading to platelet functional changes. Whether a transmembrane Na^+ flux and/or a net increase in platelet Na^+ are required for ADP-activation is presently unknown.

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